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# Design, Synthesis, and Biological Evaluation of Conformationally Constrained Analogues of Naphthol AS-E as Inhibitors of CREB-**Mediated Gene Transcription**

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Supporting Information

ABSTRACT: Cyclic AMP response element binding protein (CREB) is often dysregulated in cancer cells and is an attractive cancer drug target. Previously, we described naphthol AS-E (1) as a small molecule inhibitor of CREB-mediated gene transcription. To understand its bioactive conformation, a series of conformationally constrained analogues of 1 were designed and synthesized. Biological evaluation of these analogues suggests that the global energy minimum of 1 is the likely bioactive conformation.

# INTRODUCTION

Cyclic-AMP response element (CRE) binding protein (CREB) is a stimulus-activated nuclear transcription factor enabling cells to respond to various extracellular stimuli.<sup>1</sup> Its DNA-binding domain, located at the C-terminus, binds the consensus CRE sequence 5'-TGACGTCA-3'.<sup>2,3</sup> Even though CREB is able to bind CRE in the absence of any posttranslational modifications, its transcription activity is not activated until its activation domain called kinase-inducible domain (KID) is phosphorylated at Ser133.4 The thus phosphorylated CREB (p-CREB) allows for its subsequent binding with transcription coactivator CBP (CREB-binding protein).<sup>5</sup> The CREB–CBP interaction is mediated by KID in CREB and KIX (KID-interacting) domain in CBP.5 A variety of protein serine/threonine kinases including protein kinase A (PKA),<sup>2</sup> protein kinase B (PKB/ Akt),<sup>6</sup> protein p90 ribosomal S6 kinase (pp90<sup>RSK</sup>),<sup>7</sup> and mitogen-activated protein kinase (MAPK)<sup>8,9</sup> could phosphorylate and activate CREB. Since these kinase signaling pathways are often deregulated in cancer cells, CREB was consistently found to be overactivated in cancer tissues from patients with non-small-cell lung cancer (NSCLC),<sup>10</sup> prostate cancer,<sup>11</sup> breast cancer,<sup>12</sup> acute myeloid leukemia, and acute lympho-blastic leukemia.<sup>13,14</sup> Thus, CREB has been proposed as an intriguing target for the development of novel cancer therapeutics<sup>15</sup> and various strategies have been pursued to identify potential small molecule modulators of CREB-mediated gene transcription.<sup>15-17</sup>

We recently described naphthol AS-E (1, Figure 1) as a small molecule inhibitor of KIX-KID interaction and CREBmediated gene transcription with potencies in the low micromolar range.<sup>18</sup> However, its bioactive conformation, which would be very useful in facilitating the design of more potent analogues, is currently unknown. In this report, a series of conformationally constrained analogues of 1 were designed and synthesized to interrogate the bioactive conformation of 1 as inhibitors of KIX-KID interaction and CREB-mediated gene transcription.





# RESULTS AND DISCUSSION

To investigate the conformational preference of 1, it was subjected to a systematic conformational search by rotating all the rotatable bonds in MacroModel. This search resulted in two alternatively hydrogen-bonded conformations 1a and 1b (Figure 1) as the energy minima. Conformer 1a, with a dihedral angle of a-b-c-d of 0.1°, is fully conjugated and adopts a coplanar conformation. On the other hand, the dihedral angle of a-b-c-d in conformer 1b is 10.6°. As a consequence, the naphthyl ring and the chlorophenyl ring are not coplanar. Counterintuitively, conformer 1b is the global minimum and is energetically slightly more favorable than conformer 1a by 2.243 kJ/mol (Figure 1). To examine which conformation might represent the biologically active one, conformationally constrained analogues 2-7 (Chart 1) were designed. Compounds 2 and 3 were designed to mimic conformer 1a, while 4-7 were designed to mimic conformer 1b.

Compound 2 was synthesized in one step by treating 1 with oxalyl chloride.<sup>19</sup> The synthesis of benzo[g]quinazolinedione 3 would require precursor 11 (Scheme 1). Therefore, the amino

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Chart 1. Structures of Designed Conformationally Constrained Compounds 2–7



Scheme 1. Synthesis of 3



group in 3-amino-2-naphthoic acid (8) was first protected by a Boc group to give acid 9, which was then coupled with 4chloroaniline to provide amide 10. Removal of Boc under acidic condition (TFA) yielded amine 11, which was then treated with triphosgene to afford analogue 3 uneventfully.

The synthesis of analogue 4 was less straightforward. Initially, it was envisioned that an  $S_NAr$  reaction between 4-chloroaniline and chloride 15 would deliver 4.<sup>20</sup> Thus, a synthetic route depicted in Scheme 2 was designed. Hydroxamide 13 was





prepared by condensing hydroxylamine with the methyl ester of acid 12. However, when 13 was treated with thionyl chloride,<sup>21</sup> isoxazole 14 was not obtained as expected. Instead, oxazolone 18 was isolated in 37% yield, whose structure is consistent with the observed positive NOE between  $H_a$  and  $H_b$ . The formation of 18 is perhaps through a process analogous to the Lossen rearrangement<sup>22</sup> of chloride 16 generated from chlorination of 13 to provide isocyanate 17, cyclization of which produced 18. The electron-rich nature of the naphthyl ring may facilitate the Lossen type rearrangement pathway instead of direct ring closure to form 14. Other reagents including carbon-

yldiimidazole<sup>23</sup> and Mitsunobu reagent  $(Ph_3P/DEAD)^{24}$  were also tried, but they both failed to give 14.

The apparent difficulty in synthesizing 14 prompted us to investigate an alternative route to make 4 as presented in Scheme 3. Thus, 3-amino-2-naphthoic acid (8) was converted

Scheme 3. Synthesis of 4-6



into fluoride **19** through a three-step sequence involving methyl esterification, diazonium salt formation, and thermal decomposition of the diazonium tetrafluoroborate. The ester group in **19** was then reduced with DIBAL-H to provide aldehyde **20**, which was then transformed into the corresponding oxime. It was found that the oxime could be oxidized by NCS to give *N*-hydroxyimidoyl chloride **21**,<sup>25</sup> which was then coupled with 4-chloroaniline to yield *N*-hydroxyimidamide **22**. Intramolecular  $S_NAr$  reaction of **22** under basic condition (K<sub>2</sub>CO<sub>3</sub>/DMF) delivered analogue **4**.

The intermediate ester 19 was also utilized to synthesize 5 and 6 (Scheme 3). Saponification of ester in 19 gave acid 23, which was then coupled with 4-chloroaniline with BOP reagent to afford amide 24. The thioamide 25 was formed by treating 24 with Lawesson's reagent. Ring closure between 25 and hydrazine<sup>26</sup> delivered analogue 5. Methylation of 5 with NaH/ MeI afforded 6. The regiochemistry of the methyl group in 6 was confirmed by the positive NOE between the methyl protons and one of the naphthyl protons, while no NOE was observed between the protons in the chlorophenyl ring and the methyl group.

Finally, 7 was prepared by a sequence of reactions presented in Scheme 4. Intermediate 26 was prepared by condensing acid 8 with formamide under solvent-free conditions.<sup>27</sup> 7 was made by an  $S_NAr$  reaction between 4-chloroaniline and chloride 27, which was derived from 26 after being treated with POCl<sub>3</sub>.

Compounds 2–7 were then evaluated for inhibition of KIX– KID interaction in vitro by a Renilla luciferase complementation assay that we recently developed (Table 1).<sup>18</sup> In this assay, 1 inhibits KIX–KID interaction with  $IC_{50} = 2.90 \ \mu M.^{18}$  2 and 3 were designed to mimic conformation 1a. Compound 3 also retains a hydrogen-bond donor as seen in 1. However, neither

# Scheme 4. Synthesis of 7



Table 1. Biological Activities of  $1-7^a$ 

compd	KIX-KID inhibition $(\mu M)^b$	CREB inhibition $(\mu M)^c$
1	$2.90 \pm 0.81$	$2.29 \pm 0.31$
2	>50	$30.84 \pm 8.40$
3	>50	>50
4	>50	$24.45 \pm 16.80$
5	$8.74 \pm 1.68$	$4.75 \pm 3.88$
6	$5.19 \pm 2.20$	>50
7	$9.46 \pm 4.15$	$18.96 \pm 6.09$

<sup>*a*</sup>All the biological activities are presented as IC<sub>50</sub> with mean ± SD of at least two independent experiments or >50 if IC<sub>50</sub> was not reached at the highest tested concentration, which is 50  $\mu$ M. <sup>*b*</sup>KIX–KID inhibition was evaluated by an in vitro Renilla luciferase complementation assay. <sup>*c*</sup>CREB inhibition refers to inhibition of CREB-mediated gene transcription in HEK 293T cells using a CREB reporter assay.

of these compounds showed any activity in inhibiting KIX-KID interaction in vitro, suggesting that conformation 1a may not be biologically relevant. This lack of activity also translates into low or no inhibitory activity against CREB-mediated gene transcription in HEK 293T cells using a CREB reporter assay.<sup>18</sup> Compounds 4-7 were designed to interrogate if conformation 1b is a potential bioactive one. Although 4 was inactive in inhibiting KIX-KID interaction, 5 with a nitrogen linkage showed only slightly reduced activity (IC<sub>50</sub> = 8.74  $\mu$ M) compared to 1. The loss of activity with 4 does not seem to be due to elimination of a hydrogen-bond donor present in 1 because the methylated 6 (IC<sub>50</sub> = 5.19  $\mu$ M) and benzo[g]quinazoline 7 (IC<sub>50</sub> = 9.46  $\mu$ M) were in the same range of activity as 1. In the cell-based CREB reporter assay, the oxygenlinked 4 had very weak activity, as expected from its lack of activity in the KIX-KID interaction assay. Compound 5, on the other hand, retained activity in inhibiting CREB-mediated gene transcription with IC<sub>50</sub> = 4.75  $\mu$ M. The intermediate potency of inhibiting KIX-KID interaction by 7 was also reflected in its medium potency in CREB transcription reporter assay ( $IC_{50} =$ 18.96  $\mu$ M). However, the methylated 6 was inactive in cellbased CREB reporter assay even though it was a reasonable inhibitor of KIX-KID interaction. This discrepancy could result from unfavorable cellular permeation and/or subcellular distribution of 6, which has a higher cLogP (cLogP = 5.298) than 5 (cLogP = 4.433). Taken together, these results suggest that the likely bioactive conformation of 1 resembles conformer 1b.

To further understand the potential reasons for the observed activity among 1, 4, and 5, these structures were geometrically optimized at the HF/6-31G\*\* level of theory in Jaguar. We found that the N1 atom in 5 (Chart 1) prefers a pyramidal configuration instead of a planar one even though it is planar

when optimized at a lower level of theory (e.g., HF/3-21G). Similar nitrogen pyramidization was also observed in other systems experimentally<sup>28</sup> and in theoretical calculations.<sup>29</sup> With these optimized structures, we then generated molecular electrostatic potential (MEP) surface for each molecule from their respective electron densities (Figure 2). The major



**Figure 2.** Molecular electrostatic potential (MEP) surfaces of 1, 4, and 5 mapped to their electron densities calculated at the HF/6-31G\*\* level of theory in Jaguar. The blue indicates electrostatically positively charged regions, while the red is negatively charged. All the surfaces are normalized from -60 to +60 kcal/mol.

difference observed among 1, 4, and 5 is located in the linkage section as highlighted in green circles in Figure 2. In both of the active compounds 1 and 5, this area is represented by positively and negatively charges surfaces. However, only electrostatically negatively charged (-40 to -50 kcal/mol) surfaces are present in the corresponding area in the inactive 4 (Figure 2). It could be that the binding site in KIX for the circled area is electrostatically negatively charged. As a consequence, 4 does not fit well in that binding site, contributing to its loss of inhibition of KIX–KID interaction. On the other hand, the mixed surfaces from 1 and 5 are well accommodated.

# CONCLUSION

A series of compounds (2-7) were designed and synthesized to mimic two alternative conformations of 1 to derive its potential bioactive conformation in inhibiting KIX-KID interaction. These compounds were then biologically evaluated for their capability to inhibit KIX–KID interaction in vitro and CREB-mediated gene transcription in HEK 293T cells. These results suggest that the energetically favored conformer **1b** may represent the bioactive conformation. Molecular modeling studies suggest that the electrostatic potential surface of the newly created heterocycle needs to be at least partially positively charged. Further investigations are underway to utilize these concepts to design more potent derivatives in inhibiting KIX–KID interaction and CREB-mediated gene transcription.

### EXPERIMENTAL SECTION

**General.** See Supporting Information for details. All final compounds were confirmed to be of >95% purity based on HPLC analysis.

**3**-(4-Chlorophenyl)-2*H*-naphtho[2,3-*e*][1,3]oxazine-2,4-(3*H*)dione (2). Compound 1 (100 mg, 0.34 mmol) was dissolved in *p*xylene (4.0 mL), and the solution was stirred for 5 min at room temperature. Oxalyl chloride (150  $\mu$ L, 1.7 mmol) in *p*-xylene (1.0 mL) was then added, and the mixture was heated at 120 °C overnight. The reaction mixture was cooled to room temperature. The precipitate was collected by filtration and washed with *p*-xylene to give a pink-white solid 17 (57 mg, 52%): mp 306–308 °C (dec). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  8.77 (s, 1 H), 8.06 (d, *J* = 8.4 Hz, 1 H), 7.96 (d, *J* = 8.4 Hz, 1 H), 7.78 (s, 1 H), 7.72 (t, *J* = 9.6 Hz, 1 H), 7.61 (t, *J* = 7.3 Hz, 1 H), 7.55 (d, *J* = 8.8 Hz, 2 H), 7.33 (d, *J* = 8.4 Hz, 2 H); <sup>13</sup>C NMR (100 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  161.3, 148.5, 148.1, 137.0, 134.8, 133.9, 131.1, 130.3, 130.1, 130.1, 129.6, 127.9, 126.9, 114.9, 112.6.

**3-(4-Chlorophenyl)benzo**[*g*]**quinazoline-2,4-(1***H*,3*H***)-dione (3).** Triphosgene (7.5 mg, 0.025 mmol) was added to a stirred solution of **11** (15 mg, 0.05 mmol) in THF (1.5 mL) at room temperature. The resulting mixture was heated under reflux for 2 h. The mixture was then cooled to room temperature and diluted with THF (5 mL). The solid was collected by filtration to give a white solid (11 mg, 69%): mp >400 °C. <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  11.67 (s, 1 H), 8.70 (s, 1 H), 8.14 (d, *J* = 7.5 Hz, 1 H), 7.95 (d, *J* = 7.7 Hz, 1 H), 7.63 (dt, *J* = 7.5, 1.3 Hz, 1 H), 7.59 (s, 1 H), 7.57 (d, *J* = 9.0 Hz, 2 H), 7.47 (dt, *J* = 7.6, 1.3 Hz, 1 H), 7.43 (d, *J* = 8.8 Hz, 2 H); <sup>13</sup>C NMR (100 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  162.2, 150.1, 136.39, 135.41, 134.7, 132.7, 131.2, 129.6, 129.6, 129.4, 128.8, 128.5, 126.7, 125.0, 115.1, 110.1; ESI-MS *m/z* 320.7 (M - H)<sup>-</sup>; HRESI-MS for C<sub>18</sub>H<sub>11</sub>ClN<sub>2</sub>O<sub>2</sub> - H, calcd 321.0425, found 321.0416.

*N*-(4-Chlorophenyl)naphtho[2,3-*d*]isoxazol-3-amine (4). K<sub>2</sub>CO<sub>3</sub> (7.9 mg, 0.057 mmol) was added to a stirred solution of 22 (6.0 mg, 0.019 mmol) in DMF (1 mL) at room temperature. The resulting mixture was then heated at 110 °C for 4 h. The mixture was cooled to room temperature, and DMF was removed in vacuo. The residue was dissolved in EtOAc (50 mL), which was then washed with H<sub>2</sub>O (2 × 10 mL) and brine (2 × 10 mL). The organic solution was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated. The residue was then subjected to column chromatography, eluting with hexanes/EtOAc (10:1 to 4:1) to give a white solid (2.7 mg, 48%): mp 218–220 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 8.12 (s, 1 H), 7.98 (d, *J* = 8.8 Hz, 1 H), 7.95 (d, *J* = 8.4 Hz, 1 H), 7.86 (s, 1 H), 7.60 (d, *J* = 8.8 Hz, 2 H), 7.56 (td, *J* = 8.4, 1.2 Hz, 1 H), 7.47 (td, *J* = 8.0, 1.2 Hz, 1 H), 7.35 (d, *J* = 8.8 Hz, 2 H), 6.60 (brs, 1 H); ESI-MS *m*/*z* 295 (M + H)<sup>+</sup>; HRESI-MS for C<sub>17</sub>H<sub>11</sub>ClN<sub>2</sub>O – H, calcd 293.0476, found 293.0470.

**N-(4-Chlorophenyl)-1H-benzo[f]indazol-3-amine (5).** A mixture of thioamide 24 (60 mg, 0.19 mmol) in anhydrous hydrazine/ DMSO (0.5/0.5 mL) was stirred at 150 °C for 1 h. The mixture was cooled to room temperature and poured into water (10 mL). The organic compound was extracted with dichloromethane (3 × 10 mL). The combined organic layers were washed with brine (2 × 10 mL), dried with Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated. The residue was purified by silica gel flash column chromatography, eluting with hexanes/ethyl acetate (4:1) to yield a yellow solid (10 mg, 18%): mp 220–221 °C. <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  11.95 (s, 1 H), 9.37 (s, 1 H), 8.62 (s, 1 H), 7.98 (d, *J* = 8.0 Hz, 1 H), 7.94 (t, *J* = 8.0 Hz, 1 H), 7.82 (s, 1 H), 7.81 (d, *J* = 8.8 Hz, 2 H), 7.44 (t, *J* = 8.0 Hz, 1 H), 7.34 (d, J = 8.8 Hz, 2 H), 7.32 (t, J = 8.4 Hz, 1 H); <sup>13</sup>C NMR (100 MHz, DMSO- $d_6$ )  $\delta$  145.2, 142.1, 139.9, 133.3, 129.5, 129.0, 127.9, 127.5, 126.3, 123.0, 122.9, 119.1, 117.9, 104.3; ESI-MS m/z 292 (M – H)<sup>-</sup>; HRESI-MS for C<sub>17</sub>H<sub>12</sub>ClN<sub>3</sub> – H, calcd 292.0636, found 292.0629.

N-(4-Chlorophenyl)-1-methyl-1H-benzo[f]indazol-3-amine (6). NaH (60% in mineral oil, 6 mg, 0.15 mmol) was added to a stirred solution of 5 (40 mg, 0.136 mmol) in DMF (0.5 mL) at 0 °C under Ar. The mixture was stirred at 0 °C for 30 min, when CH<sub>3</sub>I (8.6  $\mu$ L, 0.136 mmol) was added. The mixture was stirred at room temperature for another 1 h and poured into cooled water (10 mL). The precipitate was collected by filtration. The yellow solid was purified by silica gel flash column chromatography, eluting with hexanes/ethyl acetate, 4:1, to yield a yellow solid (19 mg, 45%): mp 121-122 °C. <sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ )  $\delta$  9.47 (s, 1 H), 8.64 (s, 1 H), 8.00 (d, J = 8.8 Hz, 1 H), 7.96 (d, J = 8.4 Hz, 1 H), 7.91 (s, 1 H), 7.83 (d, J = 8.8 Hz, 2 H), 7.54 (t, J = 8.4 Hz, 1 H), 7.37 (d, J = 8.8 Hz, 2 H), 7.32 (t, J = 8.0 Hz, 1 H), 3.98 (s, 3 H); <sup>13</sup>C NMR (100 MHz, DMSO-d<sub>6</sub>) & 143.8, 141.3, 139.3, 132.9, 129.1, 128.6, 127.3, 126.9, 126.1, 122.7, 122.6, 119.1, 117.7, 117.5, 103.1, 35.3; ESI-MS m/z 305.9 (M - H)<sup>-</sup>; HRESI-MS for C<sub>18</sub>H<sub>14</sub>ClN<sub>3</sub> - H, calcd 306.0793, found 306.0785.

*N*-(4-Chlorophenyl)benzo[g]quinazolin-4-amine (7). A mixture of 4-chlorobenzo[g]quinazoline (27) (50 mg, 0.23 mmol), 4-chloroaniline (36 mg, 0.28 mmol), and DMAP (29 mg, 0.23 mmol) in *p*-xylene (1 mL) was stirred at 140 °C for 10 min. The mixture was cooled to room temperature. Evaporation of *p*-xylene resulted in a residue which was subjected to silica gel flash column chromatography, eluting with hexanes/ethyl acetate (2:1) to yield a yellow solid (31 mg, 44%): mp 251–252 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 10.26 (s, 1 H), 9.32 (s, 1 H), 8.67 (s, 1 H), 8.47 (s, 1 H), 8.21 (d, *J* = 8.8 Hz, 1 H), 8.19 (d, *J* = 8.4 Hz, 1 H), 8.08 (d, *J* = 8.8 Hz, 2 H), 7.42 (t, *J* = 7.2 Hz, 1 H), 7.54 (d, *J* = 8.8 Hz, 2 H); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ 158.6, 154.4, 145.5, 138.6, 135.9, 131.4, 129.3, 128.9, 128.5, 128.3, 128.0, 126.8, 125.5, 124.3, 123.7, 115.5; ESI-MS *m*/*z* 303.9 (M – H)<sup>-</sup>; HRESI-MS for C<sub>18</sub>H<sub>12</sub>ClN<sub>3</sub> + H, calcd 306.0793, found 306.0805.

# ASSOCIATED CONTENT

#### **S** Supporting Information

Experimental procedures for synthesis of and characterization data for 9-11, 19-20, and 22-27; details of the biological assays and molecular modeling. This material is available free of charge via the Internet at http://pubs.acs.org.

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#### Notes

The authors declare no competing financial interest.

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#### ABBREVIATIONS USED

CRE, cyclic AMP response element; CREB, cyclic AMP response element binding protein; CBP, CREB-binding protein; KID, kinase-inducible domain; KIX, kinase-inducible domain interacting; p-CREB, phosphorylated cyclic AMP response element binding protein; PKA, protein kinase A; PKB, protein kinase B; MAPK, mitogen-activated protein kinase; pp90<sup>RSK</sup>, protein p90 ribosomal S6 kinase; NSCLC,

non-small-cell lung cancer; NOE, nuclear Overhauser effect; MEP, molecular electrostatic potential;  $S_NAr$ , nucleophilic aromatic substitution

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